CONCISE REPORT

Megakaryocyte Colony Formation From Human Bone Marrow Precursors

By W. Vainchenker, J. Bouguet, J. Guichard, and J. Breton-Gorius

We report the growth in plasma clot culture of megakaryocyte colonies from adult bone marrow cells with the use of four different sources of erythropoietin (Ep) as the stimulating factor. A major proportion of the megakaryocyte colonies (75%) were pure, while the others were mixed, involving erythroblasts and megakaryocytes. Ultrastructural studies have shown that the maturation of megakaryocytes was essentially normal and that platelet shedding occurred. Megakaryocyte colony formation required a large number of plated cells (greater than $3 \times 10^5$/ml). In the absence of erythropoietin, rare spontaneous megakaryocyte colonies could be observed, while no erythroid colonies were present. However, erythropoietin induced a fivefold increase in the total number of colonies. These data suggest that erythropoietin is involved in the differentiation of human megakaryocytes, but that it does not act alone, since another factor related to the number of seeded cells appears essential for the formation of human megakaryocyte colonies.

MURINE MEGAKARYOCYTE (MK) colonies can be grown in semisolid media using several sources of stimulating factors, including erythropoietin (Ep). Using Ep, it was recently possible to obtain megakaryocyte colonies from the human precursors present in the blood of fetuses, neonates, and adults and also in fetal livers. We presently report MK colony formation from adult bone marrow precursors and compare the results with those obtained from blood. In addition, we have analyzed the role of Ep in the proliferation and maturation of MK in vitro.

Our preliminary results have appeared in abstract form.

MATERIALS AND METHODS

Bone marrow samples (2–3 ml) were collected from 10 patients by aspiration into heparinized syringes. The patients exhibited various diseases: lymphoma, Hodgkin disease, or coma due to neurologic disorders. However, they had normal hematologic parameters and their bone marrows did not show any abnormal cells. The plasma clot technique described for the cloning of murine MK precursors was used in a slightly modified form: α medium was used instead of NCTC 109 and MEM Eagle; human AB serum was substituted for fetal calf serum. Each batch of AB serum was obtained from a normal blood donor and heat inactivated at 56°C for 30 min; it was then tested by examination of its effect on the growth of human erythroid bursts and MK colonies. In these experiments, the same batch of human serum was used.

Light density cells were separated by Ficoll-metrizoate centrifugation at 400 g for 40 min at room temperature (Lymphoprep, Nyegaard, Oslo, Norway: density: 1.077 g/ml).

The cellular ring was collected and washed three times in cool α medium at 200 g. The cells were then plated at a concentration of $10^3–7 \times 10^5$ cells in a 1-ml volume in 35-mm Petri dishes (Falcon), at least in duplicate. Incubation was then performed in the presence of 3% CO₂ in air for 7–14 days in a fully humidified atmosphere at 37°C.

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Submitted March 20, 1979; accepted June 7, 1979.

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MEGAKARYOCYTE COLONIES FROM HUMAN MARROWS

Three Ep preparations of low purity were used: a sheep plasma Ep (Step III, Connaugh Laboratories, Toronto, Canada), a human urinary Ep (Los Angeles, pool M 15 Ta LSL), a crude serum from anemic mice, and highly purified Ep (70,000 U/mg protein), a gift from Dr. Goldwasser. These Ep preparations were used at concentrations ranging from 0 to 3 U/ml; 3 U/ml was the usual dose.

Erythroblast colonies were identified by revelation of the pseudo-peroxidase activity of hemoglobin. MK were identified by cytologic examination using Harris hematoxylin counterstaining.

For ultrastructural studies, the fibrin network was digested with pronase at 0.1%. The pellet was subsequently treated in order to reveal platelet peroxidase and was then prepared for electron microscopy (EM).

RESULTS

The first MK colonies appeared at day 7 or 8 of culture; their number peaked between day 10 and 12. Subsequently, the MK progressively disappeared. At day 7 or 8 only, single MK could be observed, but later, each colony involved several MK, each consisting of up to 30 typical MK (Fig. 1, inset A). The size of these colonies approximated that of an erythroid burst. Most of the colonies consisted entirely of MK; a few (15%–35%) were mixed and involved both MK and erythroblasts.

The number of colonies is indicated in Table 1. MK colonies were observed with an average frequency of 1/5500 plated cells, but these showed great individual variability.

Fig. 1. (Inset A) Megakaryocyte colony in the plasma clot at day 12 (stained with hematoxylin, observation at low magnification). Six large, polylobulated cells are seen dispersed. Many small cells cannot be identified, however, some of them resemble polymorphonuclear leukocytes. (Inset B) One megakaryocyte examined by electron microscopy. The polylobulated nucleus contains a nucleolus. The dense reaction product in the nuclear envelope indicates the presence of platelet peroxidase. The cytoplasm contains numerous organelles with the exception of the peripheral blebs (×1700). Enlargement of a section shows the nucleus (N) and platelet peroxidase localization (arrows). The demarcation membranes (DM), mitochondria (Mi), numerous α-granules (α-Gr), and a cluster of glycogen particles (Gly) are seen but are not regularly distributed (×23,500).
Table 1. Comparison in Ten Patients of the Numbers of Megakaryocyte and Erythrocyte Colonies in
the Presence or the Absence of Ep (the Ep Preparation was a Crude Serum From Anemic Mice)

<table>
<thead>
<tr>
<th>With Ep 3 U/ml</th>
<th>Without Ep</th>
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<tbody>
<tr>
<td>Megakaryocyte Colonies</td>
<td>Burst Colonies</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>120</td>
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<tr>
<td>3</td>
<td>155</td>
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<td>60</td>
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<tr>
<td>9</td>
<td>70</td>
</tr>
<tr>
<td>10</td>
<td>76</td>
</tr>
</tbody>
</table>

Mean value: 95 ± 13.2 237 ± 32.8 17.2 ± 3.25 0

ND, nondetermined.

Fig. 2. Platelet shedding (culture at day 13). The indented nucleus (N), surrounded by a thin rim
of cytoplasm, appears separated from several platelets. The platelet peroxidase reaction is present
in the perinuclear space and in the endoplasmic reticulum of the megakaryocyte as well as in a
platelet (arrows) (×14,200). (Inset) This elongated shape of the megakaryocyte is strongly sug-
gestive of platelet shedding.
variation (1/2900 to 1/8100). Only a very rough correlation existed between the number of erythroid bursts and MK colonies for the same subject (p = 0.10).

Electron microscopic examination showed that MK were of large size (Fig. 1, inset B) and that maturation was essentially normal in most of them, with the presence of demarcation membranes, α-granules, glycogen, and platelet peroxidase (Fig. 1). However, the distribution of these organelles was slightly heterogeneous. The possibility of platelet shedding was suggested on the basis of the elongated shapes of some MK at the light microscopic level (Fig. 2, inset) and by the observation of megakaryocyte fragments on electron microscopy (Fig. 2).

Figure 3 shows the influence of the dose of Ep on the growth of MK from a single sample. Similar results were obtained with the crude serum from anemic mice, as with the poorly purified human urinary Ep and with the highly purified Ep. The sheep plasma Ep was not tested. In 3 other experiments, no difference could be detected between the Ep from sheep plasma and highly purified Ep when employed at the same concentrations (respectively, 102 ± 17 and 112 ± 15 for 5 × 10^5 plated cells at 3 U/ml).

Spontaneous MK colonies were observed that reached 20% of the maximum plating efficiency in the absence of erythroid colonies (CFU-E or BFU-E) (Table 1). A significant difference in the number of MK colonies grown in the presence

![Graph showing the relationship between the number of megakaryocytic colonies and the concentration of three different erythropoietins for 5 × 10^5 seeded cells.](image)

Fig. 3. Relationship between the number of megakaryocytic colonies and the concentration of three different erythropoietins for 5 × 10^5 seeded cells.
and in the absence of Ep was observed (0.02 < α < 0.01 by test). In contrast, a good correlation was found between the number of colonies in the presence and in the absence of Ep (p < 0.001). With increase in the concentration of Ep, the number of MK colonies reached a plateau between 1.5 and 2 U/ml; however, the number of MK in each colony was not modified by the dose of Ep.

The influence of the number of plated cells is shown in Fig. 4. Thus, with less than 3 x 10^5 seeded cells and in the presence of Ep, no MK colonies could be grown, although erythroid bursts were present. For numbers of plated cells ranging from 4 x 10^5 and 7 x 10^5, a linear relationship was found.

**DISCUSSION**

Using the plasma clot technique in the presence of Ep, MK colonies could be grown from human bone marrow precursors when a large number of cells (more than 3 x 10^6) was plated. Similar results were obtained with different sources of Ep, whether purified or not. Thus, Ep acts as a stimulating factor for MK growth. However, in the absence of Ep, colonies could be observed but in decreased numbers. This last result strongly suggests that another factor is present in the culture, since no erythroid colonies could be grown under these conditions. The fact that a large number of seeded cells is necessary for the induction of differentiation towards MK favors the view that this factor is of cellular origin. In the mouse, Williams et al. have shown that a bone-marrow-conditioned medium favored MK maturation, thereby suggesting that some marrow cells are implicated in the production of this factor. A large number of seeded cells also increased the size of the murine MK colonies. This factor may be the equivalent in vitro of thrombopoietin described in vivo.
At the present time, the clonal origin of the mixed colonies involving erythroblasts and MK cannot be proven in culture derived from marrow precursors or from blood precursors. One can note however that these mixed colonies represent a similar percentage (15%-35%) of the total number of MK colonies in cultures from blood or from bone marrow. However, the total number of precursors is some sixfold greater in the marrow than in adult blood.

Two hypotheses can be proposed to explain the presence of single MK at day 7 or 8. First, that they represent the beginning of the development of one colony in which the other MK are still unidentified, or secondly, that they are really isolated and may derive from a different progenitor equivalent to CFU-E. In the absence of a specific and early marker of MK at the light microscopic level, this problem remains unsolved.

Although the present data represent the beginning of the investigation in man, the MK cultures may provide a powerful tool for study of the regulation of megakaryocytopoiesis.

REFERENCES


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